NUTRITIONAL ATTRIBUTION OF DARK CHOCOLATE

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

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SUBMITTED BY

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This is to certify that **Ms. Shawna Sen**, a student of **Master of Science in Biotechnology** Batch **2023 – 2025**, has successfully carried out her dissertation entitled: NUTRITIONAL ATTRIBUTION OF DARK CHOCOLATE.

The dissertation work was undertaken as a part of the curriculum in partial fulfillment of the requirements for the award of the degree of **Master of Science in Biotechnology** under the auspices of **Jadavpur University**.

This research work was carried out **from 01.01.2025 to 31.05.2025** under the supervision of **Prof. Runu Chakraborty**, **a professor** in Jadavpur University of Food Technology & Biochemical Engineering. The work embodied in this dissertation is original and has not been submitted previously, in part or in full, to any other university or institution for the award of any degree or diploma.

Her work was found to be satisfactory and in accordance with the prescribed guidelines of the university.

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DECLARATION

I hereby declare that the work which is being presented in this dissertation entitled "Nutritional attribution of Dark Chocolate" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology is an authentic record of my own work carried out during the period from 01.01.2025 to 31.05.2025 under the supervision of Dr. Runu Chakraborty, Department of FOOD TECHNOLOGY AND BIOCHEMICAL ENGINEERING.

I acknowledge that I have read and understood the UGC regulations, 2018 (*Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institution*).

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I further declare that no portion of the dissertation or its data will be published without the Institute's or supervisor's permission. I have not previously applied for any other degree or award using the topics and findings described in my dissertations.

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TO WHOM IT MAY CONCERN

This is to certify that the project report titled " Nutritional attribution of dark chocolate", submitted by Ms. Shawna Sen (Registration No. - 230020110795 Enrolment No – 2322210002011) to the Department of Microbiology/Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by him/her for the partial fulfillment of the requirements for the Master of Science degree.

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ABSTRACT

Dark chocolate, which contains bioactive substances such as polyphenols and methylxanthines, has evolved from a treat to a functional food with numerous health advantages. Its consumption is linked to a lower risk of vascular endothelial diseases, diabetes, oxidative stress, obesity, a healthier lipid profile and improved cognitive function. This study goes over the analytical methods used to determine dark chocolate samples' nutritional profile and antioxidant properties. Soxhlet extraction for fat analysis, calculation by difference for carbohydrate content, oven drying for moisture analysis, incineration for ash content, Folin-Ciocalteu assay for total polyphenol content, aluminium chloride method for total flavonoid content, DPPH assay for free radical scavenging activity, and FRAP assay for ferric reducing antioxidant power are among the techniques used. The study found strong associations between cocoa content and macronutrient/antioxidant profiles. Higher cocoa content boosted protein, ash, polyphenol, flavonoid, and antioxidant activity (DPPH and FRAP) while decreasing fat, carbohydrate, and moisture levels. These findings emphasize the nutritional superiority of dark chocolate with greater cocoa percentages, emphasizing cocoa solids' significant contribution to its bioactive content and possible health advantages. This study demonstrates that higher cocoa content in dark chocolate correlates with higher amounts of protein, ash, polyphenols, and flavonoids and increased antioxidant activity. Conversely, larger cocoa percentages result in lower fat and carbohydrate intake. These findings highlight the better nutritional profile and functional potential of high-cocoa dark chocolate, supporting its inclusion as a health-promoting food when consumed in moderation as part of a balanced diet.

Keywords: Dark chocolate, Cocoa content, Nutritional profile, Polyphenols, Flavonoids, Antioxidant activity, Functional foods, Bioactive compounds.

1. INTRODUCTION

Due to its many health advantages, dark chocolate has been popular for many years. It includes several health-promoting elements (bioactive components, such as procyanidins, theobromines, flavonoids, polyphenols, and vitamins and minerals) that positively affect human immunological function. It offers protection against heart disease, several malignancies, and other brain-related conditions like Parkinson's and Alzheimer's illnesses [1]. Dark chocolate is regarded as a functional food because of its anti-inflammatory, anti-diabetic, and anti-microbial qualities. It is also well known for helping people control their weight and healthily change their lipid profile. However, several minerals (polyphenols, flavonoids, flavan-3 ol, ascorbic acid, and thiamine) are lost during the preparation of dark chocolate. Therefore, fortification would be a good way to increase the total nutrients and make dark chocolate self-sufficient. Therefore, compiling all experimental research on dark chocolate fortification is the primary goal of this review study. Fruits (mulberries, chokeberries, and elderberries), spices (cinnamon), peanut oil, phytosterols, probiotics (mostly Lactobacillus, bacillus spices), prebiotics (inulin, xanthan gum, and maltodextrin), flavonoids, flavan-3-ols, etc. were among the components employed for the fortification [2]. Those fortifications aimed to decrease the overall calorie content while increasing the concentration of vital fatty acids and total antioxidants. By altering its bitter flavour, fortification was occasionally used to enhance physical characteristics, including viscosity and rheological qualities, and increase consumer acceptance [3].

Dark chocolate, derived from the fermented, dried, and roasted seeds of the *Theobroma cacao* tree, has evolved from a luxury indulgence into a nutritional and biomedical interest subject. Unlike milk chocolate, dark chocolate contains a significantly higher proportion of cocoa solids and typically less added sugar and dairy fat [4]. This composition influences its taste and texture and contributes to a more potent nutritional profile. Increasingly, dark chocolate is being studied not just as a food item but as a functional food—defined as a food that offers health benefits beyond fundamental nutritional value [5].

Dark chocolate is nutritionally rich due to its high concentration of bioactive ingredients. It is exceptionally high in polyphenols, particularly flavonoids like flavan-3-ols and procyanidins, which have potent antioxidant qualities [6]. These antioxidants are essential for neutralizing free radicals, which may reduce oxidative stress and minimize the risk of chronic diseases like cardiovascular disease, neurological problems, and some types of cancer [7]. Furthermore, dark chocolate contains critical minerals such as iron, magnesium, copper, and manganese, all of which play essential roles in physiological processes

such as oxygen transport, enzyme reactions, nerve function, and bone health. It also contains some dietary fibre, which may help digestion and satiety [8].

In addition to its micronutrient content, dark chocolate contains trace amounts of stimulants, including caffeine and theobromine [9]. However, these substances are known to improve mood and cognitive performance to a lower extent than more concentrated sources such as coffee or tea [10]. Emerging research suggests that moderate consumption of dark chocolate may enhance insulin sensitivity, blood pressure management, and lipid profiles, highlighting its potential significance in preventive nutrition [11].

This section will go deeper into the nutritional components of dark chocolate, looking at their individual and synergistic functions in health promotion and illness prevention [12]. The nutritional attribution of dark chocolate will be investigated in the context of modern dietary patterns and evidence-based health recommendations, using a rigorous investigation of its macro- and micronutrient content and phytochemical components [13,14].

Dark chocolate contains alkaloids, including theobromine and caffeine, which have modest stimulant properties and may affect cognitive performance, mood, and alertness. Theobromine, in particular, is recognized for its vasodilatory and diuretic effects, which add to the cardiovascular benefits of eating dark chocolate. Some recent evidence suggests that dark chocolate can improve insulin sensitivity, modulate inflammatory indicators, and enhance neuroprotective processes, implying a wider range of health benefits. While dark chocolate is calorically dense and should be consumed in moderation, its unique combination of bioactive phytochemicals and vital elements distinguishes it as a functional food with substantial medicinal potential. However, factors such as cocoa content, processing processes, and added additives can all impact the product's nutritional value and health benefits [15,16].

Chocolate is a popular and delicious treat for people of all ages. Its global consumption rate continues to rise year after year. According to consumption figures, Switzerland had the highest chocolate consumption. According to a 2017 poll, Swiss people have a long history of eating chocolate. Austria ranked second after Switzerland in terms of per capita chocolate consumption. Germany, Ireland, the United Kingdom, Sweden, Estonia, Norway, and Poland are among the countries with the highest per capita chocolate consumption [17].

Chocolate is extremely popular among consumers due to its delicacy and health advantages. The usage of chocolate first began in Mesoamerica. The ancient Aztecs utilized chocolate to treat a variety of diseases. Chocolate also improves general longevity, sexual appetite, and fertility. Chocolate contains cocoa, which contributes to its health advantages. Cocoa was first grown 5300 years ago in Central America by the Mayo-Chinchipe people. The cocoa bean's principal antioxidant phytonutrients are polyphenols and flavonoids. Polyphenols account for 10% of the cocoa bean's dry weight. The most common flavonoids in cocoa powder are catechins, anthocyanins, and proanthocyanidins [18]. Cocoa beans (Theobroma cacao) contain 50-57% lipid, primarily known as cocoa butter. Cocoa butter is a key component of dark chocolate. Cocoa butter's principal ingredients are 33% oleic acid, 25% palmitic acid, and 33% stearic acid. Cocoa is widely popular due to its numerous health advantages. Cocoa consumption is associated with a 50% reduction in cardiovascular mortality. Chocolate is a functional food since it helps avoid

degenerative diseases such as diabetes, obesity, Alzheimer's disease, and others. When health difficulties come to mind, we should make the appropriate choice.

Dark chocolate is a better choice than milk chocolate since it contains more polyphenols and flavonoids. Dark chocolate contains five times more polyphenols and flavonoids than milk or white chocolate. Milk and white chocolate contain significantly more fat (30 g fat per 100 g) and sugar (52 g per 100 g). Henderson and Hudson (2019) first recognized the advantages of cocoa beverages before 1000 B.C. Chocolate-based drinks improve overall vitamin D and calcium levels in humans.

Additionally, it boosts IFN-γ levels, which increases immunity. The chocolate drink also raises the BMI. As a result, it can prevent the underweight problem and has been successfully employed in the malnourished community to prevent malnutrition [19,20].

Chocolate manufacturing is a multistep process that includes fermentation, drying, roasting, nib grinding and refining, and tempering cocoa beans. During this lengthy treatment, nutritional loss is significant. Polyphenol content decreases about tenfold from its original form. Some flavonoids disappear completely with the production of Maillard products [21]. As a result, the antioxidant composition varies between raw materials and end products. Approximately half of the epicatechin is lost after drying. A higher drying rate produces acetic acid, which contributes to undesirable flavours, whereas too slow drying results in increased mould development and an absence of appropriate colour in the chocolate. Anthocyanin degrades during fermentation due to hydrolysis and polymerization of condensed tannins. Epicatechin, polyphenol, and procyanidin levels fall dramatically during fermentation. Even anthocyanidins become undetectable during fermentation. Fortification is an excellent strategy to compensate for these significant phytonutrient losses [22]. Its medicinal value will increase if dark chocolate is fortified with additional beneficial micronutrients.

Micronutrient insufficiency is prevalent in India. Fortification is a good solution to this problem because it increases vitamin intake. To the best of our knowledge, no analogous review exists on the effect of reinforced dark chocolate on human health. The current review article covers the experimental studies that focus on different approaches to fortification [23]. It also presents the positive impact of dark chocolate fortification with different micronutrients for raising overall immunity and nutritional status by preventing micronutrient deficiency diseases in the human community. Dark chocolate is a nutritious food item widely used as a dessert by people of all ages. As a result, dark chocolate could be an excellent choice for fortification. After processing, nutrient losses must be compensated, and fortification is a critical method for making a more abundant supply of essential nutrients [24]. Dark chocolate has already been fortified with various vitamins

and beneficial substances. The current research investigates the positive association between dark chocolate fortification and disease reduction. Finally, it highlights the therapeutic value of newly produced dark chocolate and its efficient application in preventing various diseases [25].

1.1. Bioactive components present in cocoa:

Cocoa, the fundamental component in dark chocolate, contains many bioactive chemicals that contribute to its health-promoting effects. These chemicals, particularly polyphenols, methylxanthines, and biogenic amines, have a variety of biological properties, including antioxidant, anti-inflammatory, cardioprotective, neuroprotective, and metabolic actions. The concentration and efficacy of these bioactive chemicals can differ depending on the cocoa bean variety, processing methods, and final product composition [26].

1.1.1. Polyphenols:

Polyphenols are the most common bioactive component in cocoa, accounting for about 12-18% of the dry weight of unfermented cocoa beans. The primary polyphenols in cocoa are:

1.1.1.1. Flavanols (flavan-3-ols):

These are the most extensively investigated cocoa polyphenols, with epicatechin and catechin being the most common. Flavanols have strong antioxidant properties, scavenging free radicals and boosting the body's endogenous antioxidant defence systems. They also promote vascular health by increasing nitric oxide production, which causes vasodilation and improves blood flow.

1.1.1.2. Procyanidins:

These oligomeric or polymeric forms of flavanols account for much of cocoa's antioxidant properties. Procyanidins have been found to lower oxidative stress, prevent LDL oxidation, and improve cardiovascular health.

1.1.1.3. Anthocyanins and other flavonoids:

However, These chemicals, in smaller levels, add to cocoa's antioxidant capability and overall health advantages.

1.1.2. Methylxanthines:

Cocoa contains two primary methylxanthines:

1.1.2.1. Theobromine:

This is the most abundant methylxanthine in cocoa. It has a modest stimulant effect, improves mood, and stimulates vasodilation and diuresis. Theobromine has also been linked to improved respiratory function and cardiovascular health.

1.1.2.2. Caffeine:

Caffeine in cocoa, although in smaller proportions than in coffee or tea, adds to its stimulatory effects on the central nervous system, including greater alertness and potential cognitive benefits.

1.1.3. Biogenic Amines:

Cocoa includes trace levels of biogenic amines, including phenylethylamine (PEA), which is hypothesized to function as a neuromodulator. PEA is sometimes called the "love chemical" because of its link to mood elevation and a sensation of well-being. However, its quick metabolism in the body restricts its systemic benefits. **1.1.4. N-acyl ethanolamines (NAEs):**

These lipid-like compounds, found in trim levels in cocoa, are thought to imitate the actions of cannabinoids by interacting with cannabinoid receptors in the brain. NAEs may have appetitesuppressant and mood-altering qualities.

1.1.5. Minerals and Other Phytonutrients:

Although not precisely bioactive, cocoa contains essential minerals such as magnesium, iron, copper, and zinc, all of which have metabolic and antioxidant activities. Furthermore, cocoa contains tiny peptides and amino acids that may have biological effects, particularly on blood pressure and inflammation.

Many of the physiological benefits of dark chocolate consumption are attributed to cocoa's rich matrix of bioactive chemicals. These chemicals work together to provide antioxidant, antiinflammatory, and cardioprotective benefits. A better understanding of these bioactives is required to evaluate cocoa and dark chocolate as food products and as possible therapeutic agents in disease prevention and health promotion [27,28].

1.2. Health Benefits of Dark Chocolate:

1.2.1. Protection against vascular endothelial disorders:

Regular eating of dark chocolate reduces the likelihood of developing high blood pressure. As a result, it delays the onset of cardiovascular diseases. Vascular endothelial dysfunction is the most potent critical event in atherosclerosis. The primary cause of vascular endothelial dysfunction is diminished nitric oxide (NO) bioactivity, which results in inefficient low-mediated vasodilation (FMD) [29]. Cocoa flavanols can increase circulating NO levels, which raises the response of FMD in conduit arteries. This effect results in a significant increase in microcirculation. Epicatechin-7-O-glucuronide is an independent predictor of vascular effects when flavonoid-rich cocoa is consumed. It also improves vascular endothelial system performance by increasing platelet function in healthy smokers 2-8 hours after consumption [30].

1.2.2. Protection from diabetes:

It increases insulin sensitivity by lowering insulin resistance. Dark chocolate promotes blood vessel health and normal blood flow. The NOS enzymes reduced NO production, which is also responsible for faulty insulin synthesis. This can lead to the development of insulin resistance. Epicatechin in cocoa increased endogenous NO generation, which stimulated PI3K signalling. PI3K signalling pathways can influence insulin activation and glucose transport in metabolic tissues up to the point of Akt activation [29]. Insulin's hemodynamic action is defined by capillary recruitment, which leads to increased glucose uptake. Cocoa polyphenol stimulates insulin synthesis in pancreatic β cells through the Akt/PI3K and ERK1/2 pathways. Procyandine, another healthy component found in dark chocolate, can lower post-prandial blood glucose levels.

Procyanidin has low bioavailability, yet it can still interact with glucose transporters. Translocation of GLUT4 to the muscle can improve central glucose elimination and insulin signalling [31].

1.2.3. Protection from oxidative stress:

It has received scientific attention for its incredible antioxidant qualities and cancer-fighting abilities. Metabolic activities continuously produce free radicals. These free radicals harm healthy cells, and eating dark chocolate reduces unneeded cell damage. Antioxidants eliminate free radicals, protecting the body from the onset of cancer and ageing. Dark chocolate stimulates

mitochondrial activity as measured by citrate synthase activity and raises glutathione levels, protecting against oxidative stress. Dark chocolate contains epicatechin, which increases nitric oxide levels and stimulates co-activator 1-alpha (PCG-1 α), a microbial biogenesis stimulant. These findings improved mitochondrial activity, protected against oxidative stress, increased cellular metabolism, and eventually improved cellular metabolism. Mitochondrial function increases the level of ATP, which stimulates skeletal function. Epicatechin plays another purpose in mitochondria. It directly suppresses the mitochondrial permeability transition pore (mPTP), protecting against cell death [30,32].

1.2.4. Amelioration of obesity:

Dark chocolate is also used to maintain weight due to its low-calorie content. Consuming dark chocolate regularly can help to decrease obesity. Cocoa polyphenols inhibit the primary stages of adiposity in preadipocytes. According to in vitro studies, cocoa polyphenol extract can inhibit dietinduced adipogenesis by reducing mitotic clonal growth, a crucial stage in DNA remodelling for gene expression during fat cell development. Specifically, two types of transcription factors (peroxisome proliferator-activated receptor gamma and CCAAT enhancer-binding proteins alpha) are blocked, resulting in reduced mRNA production of fatty acid synthase in these cells [31].

1.2.5. Maintenance of healthy lipid profile:

Consuming moderate amounts of dark chocolate will help to maintain healthy blood cholesterol levels. Cocoa reduces the number of cholesterol receptors, hence inhibiting cholesterol absorption and production. The cocoa product lowers LDL and total cholesterol but has no meaningful effect on HDL levels [32]. Cocoa polyphenols can inhibit lipid peroxidation in the liver and serum, lowering malondialdehyde formation. However, cocoa fibre does not affect the liver's glutathione levels.

1.2.6. Stimulate brain function:

Cocoa is also thought to energize the human body. So, the cocoa-containing product is typically ingested before indulging in strenuous activity. Another key benefit of dark chocolate is improved brain function. Dark chocolate increases blood flow to the heart and brain [30]. The many chemical compounds in dark chocolate promote cognitive performance while positively modulating mood swings. Dark chocolate has a beneficial effect on theobromine and nerve growth factor levels in plasma.

1.2.7. Anti-inflammatory effect:

Several experimental research have indicated that dark chocolate has anti-inflammatory properties. It increases mRNA expression, particularly the anti-inflammatory cytokine IL10, by inhibiting the pro-inflammatory stress response. Cocoa also has direct effects on immunological cells. As a result, it can affect both innate and acquired immunity. Consuming cocoa regulates the secretion of inflammatory chemicals by macrophages and other leukocytes [32].

2. OBJECTIVES

I have selected dark chocolate as the focus of my study because of its rich nutritional profile and the presence of health-promoting compounds such as polyphenols, flavonoids, and antioxidants. The main objective of my research is to explore and understand the nutritional composition of dark chocolate, with a special focus on its antioxidant and physical properties that contribute to its overall health benefits. With the increasing popularity of dark chocolate as a functional food, it was felt that it was essential to look beyond its sensory appeal and investigate its nutritional makeup through detailed laboratory-based analysis. The study aims to conduct a comprehensive dietary evaluation of dark chocolate by assessing its proximate composition, including parameters like moisture content, ash content, total protein, fat, and carbohydrate levels. These factors define any food product's overall energy value, stability, and quality, key aspects of the food industry.

Therefore, this experimental study investigated its core nutritional parameters through standard biochemical tests. In the laboratory, in-vitro tests were conducted to estimate moisture content, reflecting the chocolate's water activity and affecting its texture and microbial stability. The ash content provides insight into the mineral content, vital for determining the inorganic nutritional components. Estimating protein content is essential as proteins are important macronutrients, and their quantity can vary depending on the formulation and processing of the chocolate. Fat estimation is fundamental in chocolate since cocoa butter forms a significant portion of its composition and influences energy density, texture, and taste. Similarly, understanding carbohydrate content helps assess the chocolate sample's energy contribution and sugar levels.

I hope this study gives me practical exposure to food analysis techniques and helps me contribute to the growing body of literature supporting healthier and more informed food consumption. In conclusion, this dissertation aims to bring a well-rounded understanding of the nutritional attributes of dark chocolate through practical lab-based assessments while aligning with my academic interest in food biotechnology and nutritional science.

3. REVIEW OF THE LITERATURE

According to Samanta et al. (2022), Dark chocolate, made from the cacao (*Theobroma cacao*) bean, has received much scientific attention recently because of its complex nutritional profile and potential health advantages. While dark chocolate has traditionally been considered a confectionary product, modern dietary science increasingly views it as a functional food with beneficial phytochemical qualities, particularly in high cocoa varieties [33]. This literature review aims to analyze the body of information regarding the nutritional components of dark chocolate and the bioactive chemicals it contains, focusing on how cocoa content, processing, and ingredient variability influence its health-related properties.

Protein is a minor but essential component in chocolate. According to [34], dark chocolate contains little protein, mainly from cocoa solids. According to research, increased cocoa content increases protein levels because cocoa powder contains more protein than other substances like sugar or

milk solids [35]. Although not a substantial source of dietary protein, including protein in dark chocolate increases its nutritional density and can help with satiety.

Chocolate contains a lot of fat, mostly from cocoa butter. It has a distinct fatty acid composition: saturated stearic acid, monounsaturated oleic acid, and saturated palmitic acid. Unlike other saturated fats, stearic acid is considered biologically neutral regarding cholesterol levels [36,37]. It discovered that, while overall fat content remains high in all chocolates, the composition of fats in dark chocolate can be more beneficial than in milk chocolate due to the larger proportion of cocoa butter and lack of additional milk fats.

The carbohydrate amount of dark chocolate varies greatly depending on the formulation. Higher cocoa content is often associated with lower sugar levels. According to [38], black chocolate with 70% or greater cocoa solids has significantly less sugar than milk or white chocolate. This makes high-cocoa dark chocolate a lower-glycemic alternative, appealing to people watching their blood sugar levels. Furthermore, decreasing sugar intake promotes polyphenol bioavailability, which sugar would otherwise reduce.

Moisture content is an important quality indicator for chocolate. [39] moisture levels fall as cocoa content increases, most likely due to the absence of hygroscopic components like sugar. Lower moisture content improves shelf life and microbiological stability. Meanwhile, ash content is a sign of mineral content, with higher values in darker chocolates indicating increased mineral density [40]. Minerals such as magnesium, iron, potassium, and calcium are rich in cocoa solids, improving dark chocolate's nutritional value.

One of dark chocolate nutrition's most extensively examined features is its polyphenol concentration. Cocoa is one of the most abundant natural sources of dietary polyphenols, particularly flavonoids like epicatechin, catechin, and procyanidins. [41] stressed that these chemicals have strong antioxidant properties, with dark chocolate having much greater amounts than milk or white chocolate. The European Food Safety Authority (EFSA, 2012) has acknowledged the health benefits of cocoa flavanols, notably in terms of vascular flexibility and blood flow. [42] discovered that the flavonoid content of dark chocolate increases with cocoa percentage, indicating that high-cocoa chocolate is more favorable to cardiovascular health. Furthermore, flavonoids have been associated to neuroprotective benefits [43], increased insulin sensitivity [44], and decreased inflammation [45]. However, the bioavailability of these chemicals varies depending on the flavonoid type, dietary matrix, and individual metabolism [46]. Dark chocolate's antioxidant capacity is mostly due to its polyphenol concentration. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay is widely employed to assess this activity. According to [47], dark chocolate has a higher antioxidant content per serving than numerous regularly consumed fruits, including blueberries and cranberries. High antioxidant activity is linked to lower oxidative stress and may help avoid chronic diseases like cancer, diabetes, and cardiovascular disease.

Numerous studies have confirmed that cocoa content is a key factor in nutritional quality. Dark chocolate with at least 70% cocoa content has more protein, fibre, minerals, polyphenols, and antioxidants but less sugar and moisture [48]. This differs from milk chocolate, which includes more sugar, less cocoa mass, and fewer beneficial ingredients.

Fermentation, drying, roasting, grinding, and conching are all phases of the cocoa bean's path to final chocolate, and each impacts nutritional makeup. Fermentation is required to produce flavour precursors, but it can destroy polyphenols by enzymatic oxidation [49]. Roasting decreases polyphenol concentration while increasing scent and flavour [50]. The conching process, which includes prolonged mixing and heating, may potentially contribute to losing heat-sensitive nutrients. Despite these losses, effective processing can save a considerable amount of beneficial chemicals. For example, moderate roasting and shorter conching periods have been demonstrated to preserve more antioxidants [51]. This emphasizes the necessity for a balance between sensory quality and nutritional preservation.

With the growing interest in functional foods, dark chocolate is being promoted for its healthpromoting benefits. A systematic review by [52] discovered that flavanol-rich dark chocolate can lower blood pressure and improve endothelial function. Furthermore, consuming 30-50g of dark chocolate daily has been linked to lower LDL oxidation, higher HDL levels, and decreased platelet aggregation [53]. Nonetheless, the energy density of dark chocolate must be addressed. Despite its healthy elements, dark chocolate is high in calories and fat, requiring moderation to avoid adverse energy balance effects. As a result, public health messages encourage small, consistent servings of dark chocolate as part of a healthy diet.

Although much study has been done, numerous gaps remain. Much research is limited to in vitro evaluations or short-term human trials, rendering long-term health consequences unclear. The methodology used to measure polyphenol content and antioxidant activity varies, complicating cross-study comparisons. Furthermore, the absorption and metabolism of cocoa polyphenols require additional investigation via pharmacokinetic research and clinical trials. Further research is needed to determine how diverse cocoa bean origins, farming practices, and post-harvest treatments affect the nutritional profile of dark chocolate [54]. Understanding individual reactions to cocoa flavonoids, which are influenced by genetics, microbiota makeup, and metabolic conditions, may further assist personalized nutrition techniques.

In conclusion, the science supports dark chocolate—particularly variations high in cocoa content—as a nutrient-dense and phytochemically potent diet. Its nutritional profile contains moderate protein, healthy fats, necessary minerals, and a fantastic assortment of polyphenols and antioxidants. While cocoa concentration and processing processes alter its dietary properties, highquality dark chocolate may provide health advantages when consumed in moderation. Future studies should focus on bridging gaps through long-term studies, standardized analytical methodologies, and bio-efficacy investigations, ultimately strengthening dark chocolate's significance in functional nutrition [55].

4. Materials and Method

4.1.Materials

i. Hot Analytical balance (±0.001 g precision) **ii.** Tongs and gloves (for safe handling of hot crucibles) **iii.** Muffle furnace (capable of maintaining 500–600°C) **iv.** Porcelain crucibles

v. Desiccator with active desiccant (e.g., silica gel) **vi.** Analytical balance
vii. Hot plate
viii. Tongs or heat-resistant gloves **ix.** Soxhlet apparatus with condenser and heating mantle **x.** Round bottom extraction flasks (pre-weighed) **xi.** Oven (for drying)
xii. Desiccator

4.2.Chemicals –

i. Reagent A (Alkaline Copper Solution): 2% Sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH)

ii. Reagent B (Copper Sulfate Solution): 1% Copper sulfate ($CuSO_4 \cdot 5H_2O$) in distilled water **iii.** Reagent C (Tartrate Solution): 2% Potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) in distilled water

iv. Lowry Reagent (Reagent D): Prepared fresh by mixing 50 mL of Reagent A, 0.5 mL of Reagent B, and 0.5 mL of Reagent C

v. Folin-Ciocalteu Reagent (1N): Commercially available, diluted to 1N with distilled water before use

vi. Bovine Serum Albumin (BSA): Standard protein solution (1 mg/mL in distilled water) **vii.** Distilled Water

viii. Petroleum ether (boiling range 40 60^0 C) or hexane (analytical grade)
ix. Whatman filter paper or extraction thimbles **x.** Anhydrous sodium sulfate (Na_2SO_4) **xi.** Methanol (80% v/v in water) **xii.** Folin–Ciocalteu reagent **xiii.** Sodium carbonate (Na_2CO_3, 7.5% w/v solution) **xiv.** Gallic acid standard (for calibration curve) **xv.** Aluminium chloride solution (10% w/v in methanol) **xvi.** Potassium acetate solution (1 M) **xvii.** Catechin standard (for calibration)
xviii. Centrifuge tubes and pipette **xix.** DPPH solution (0.1 mM in methanol) **xx.** Ascorbic acid (for standard curve or positive control)
xxi. Acetate buffer (300 mM, pH 3.6) **xxii.** TPTZ solution (10 mM in 40 mM HCl) **xxiii.** Ferric chloride ($FeCl_3 \cdot 6H_2O$, 20 mM solution) **xxiv.** UV-Visible spectrophotometer **xxv.** 300 mM acetate buffer (pH 3.6)
xxvi. 10 mM TPTZ solution (prepared in 40 mM HCl) **xxvii.** 20 mM $FeCl_3 \cdot 6H_2O$ solution

5.1. Moisture analysis:

This section describes the methods used to determine the moisture content of dark chocolate samples. Moisture content is an essential criterion in evaluating food quality and shelf life since it affects microbiological stability, texture, and storage qualities. The moisture analysis was carried out using the gravimetric oven-drying method, a widely acknowledged food analysis process as stated by the AOAC (Association of Official Analytical Chemists) [62,63].

Dark chocolate samples were collected from local retail stores, reflecting various commercial brands and cocoa contents. Before analysis, samples were held in airtight containers at room temperature to prevent any changes in moisture content. To guarantee a consistent texture, each chocolate sample was finely grated and homogenized before moisture analysis. Each measurement included approximately 5.0 grams of the prepared sample [63]

Moisture content is calculated by measuring the weight loss of a food sample after heating in a drying oven at a constant temperature. The weight loss represents the amount of water evaporated from the sample.

- **Procedure:**

i. A clean, dry cubicle was weighed and labeled as W1. A 5.0 g homogenized chocolate sample was inserted in the cubicle, and the combined weight was recorded as W2.

ii. The crucible containing the sample was placed in a drying oven set at 105°C for 4 to 6 hours. The drying period was sufficient to guarantee that all moisture was removed.

iii. After drying, the crucible was placed in a desiccator to chill for 30-45 minutes to avoid moisture reabsorption from the atmosphere.

iv. After cooling, the crucible was reweighed, with the final weight recorded as W3.

- **Calculation:** The percentage of moisture content was estimated using the following formula:

Moisture (%) = [(W2 – W3) * 100] / (W2 – W1) Where:

W1 = Weight of Empty Crucible (g)

W2 = Weight of crucible + Sample before drying (g)

W3 = Weight of crucible + Sample after drying (g)

5.2. Ash analysis:

This section describes the methodology used to determine the ash content of dark chocolate samples. Ash represents a food's total mineral content and is an important characteristic in nutritional and quality evaluation. The analysis was carried out in a muffle furnace using the dry ash method, in accordance with conventional food analysis protocols such as those suggested by the AOAC [64,65].

Dark chocolate samples were obtained from a variety of local retail locations to guarantee a diverse range of commercial items with varying cocoa content. All samples were kept in sealed containers at room temperature to avoid contamination or moisture changes until being analyzed. Each sample was thoroughly grated and homogenized to ensure consistency. Approximately 5.0 grams of each prepared sample were used to determine ash content.

The dry ash process incinerates the organic content of a food sample at high temperatures in a muffle furnace, leaving a residue of inorganic minerals. The weight of this residue is used to determine the ash content.

- **Procedure:**

  i. A clean, dry porcelain crucible was weighed and identified as W1. Approximately 5.0 g of the homogenized dark chocolate sample was added to the crucible. The crucible containing the sample was then weighed and identified as W2.
  ii. To prevent excessive smoking and sample loss, slowly heat the crucible on a hot plate until completely blackened.
  iii. The crucible was placed in a muffle furnace at 550°C for 5-6 hours, or until a light grey or white ash formed, indicating complete combustion of organic materials.
  iv. After ashing, the crucible was carefully taken from the furnace and cooled in a desiccator for 30-45 minutes to prevent moisture absorption from the atmosphere.
  v. The crucible containing the ash was then weighed and identified as W3.

- **Calculation:** The ash content was determined using the following formula: **Ash (%) = [(W3 – W1) * 100] / (W2 – W1)** Where:

W1 = Weight of Empty Crucible (g)
W2 = Weight of crucible + Sample before ashing (g)
W3 = Weight of crucible + Sample after ashing (g)

5.3.Colour analysis:

Colour is one of the first things people notice when looking at any food, including dark chocolate. When I started working with dark chocolate samples in the lab, I realized that colour plays a bigger role than appearance. It gives us clues about the quality, composition, and even the processing methods used to make the chocolate. That's why I felt including colour analysis in my study was essential. In dark chocolate, the colour can vary from deep brown to almost black, depending on the cocoa content, the level of roasting of the cocoa beans, and the presence of other ingredients like milk solids or sugar. A darker shade usually means higher cocoa content, which can be related to more antioxidants and flavonoids. On the other hand, uneven or dull colour may sometimes

indicate poor processing, bloom formation, or improper storage. These are small things that consumers may not understand, but they matter a lot regarding product quality.

Doing a lab colour analysis helps objectively measure these visual differences using instruments instead of just depending on the eyes. For example, using colourimeters or visual grading against standards can help detect if there are any unwanted changes due to oxidation or fat bloom. I found this interesting because even though chocolate may look fine to the naked eye, these tests can pick up on changes that show early spoilage or quality loss. Also, colour is directly linked to consumer acceptance. If the chocolate doesn't have that rich, smooth brown look, many might assume it's not fresh or tasty, even before tasting it. So, from both a nutritional and a marketing point of view, colour matters. As someone studying biotechnology and interested in food quality, I think connecting physical appearance with scientific data is essential to understanding the product thoroughly.

- **Colour estimation -**

The colour profile analysis was determined by the Hunter Lab colour measurement system (Color Flex 45/0, D 65, 10° observer; Hunter Associates Laboratory Inc. Reston, VA, USA). A 3.5 cm white standard plate (L* = 93.49; a* = −1.07; b* = 10.6) calibrated the instrument. Samples were contained in optical glass cells 3.5 cm long and 6 cm in diameter. The results were expressed in L* lightness (0; black to 100; white), a* redness (−; green to +; red), and b* yellowness (−; blue to +; yellow) values. Chrome (C*) was considered a quantitative attribute of colourfulness to determine the degree of difference of a hue compared to a grey colour with the same lightness, C* = (a2 + b2)1/2. Hue angle (h*) was used to define the difference of colour regarding grey color. h* = 1/tan (b*/a*). The equation and the color measurement followed the procedures outlined by Granato and Ellrndersen.

- **Procedure** –

To analyze the colour of dark chocolate, I followed a visual and instrumental approach. The aim was to observe the appearance of the chocolate sample and also check for any signs of colour variation that might indicate changes in quality. Here is the step-by-step procedure I followed.

I. First, I made sure the dark chocolate sample was at room temperature and free from any moisture or surface irregularities. I used a clean, dry knife to break the chocolate into flat, uniform pieces for consistent observation.
II. I placed the chocolate samples on a clean white background (like a white paper sheet) under good natural light. I observed the surface colour, glossiness, and any visible defects like whitish patches (fat bloom) or dullness. I also noted the overall colour tone—whether it appeared dark brown, reddish-brown, or had a greyish cast.

In the lab, I used a **colorimeter** -

a. **L-** represents lightness (0 = black, 100 = white).
b. **A-** indicates red-green values (positive = red).
c. **B-** shows yellow-blue values (positive = yellow).
I placed the chocolate sample on the measuring plate and took readings in triplicates for accuracy. The average values were noted for analysis.

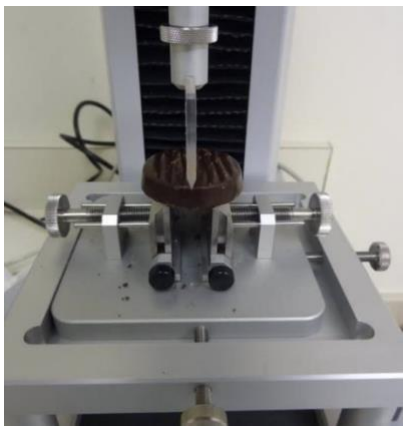
III.

I recorded the colour values and compared them to standard values for commercial dark chocolate. Any major differences could indicate processing variations or storage effects. If visible colour defects were present (like blooming), I noted them along with possible reasons such as temperature fluctuation or improper tempering.
Based on the results, I interpreted whether the sample was of good visual quality. Darker shades often suggest higher cocoa content, while lighter or patchy colours may point to fat migration or oxidation. The consistency of colour across the sample also indicated uniform processing.

5.4. Texture analysis :

The texture of dark chocolate tells us a lot about how it has been processed and what it contains. For example, chocolate's firmness or "snap" is closely related to its fat content, especially cocoa butter, which is a significant component. Well-tempered chocolate with the right amount and type of fat gives a clean snap and smooth melt in the mouth. This feels good while eating and indicates a proper balance of nutrients like fats and solids. If the texture is too soft, sticky, or crumbly, it can mean that the fat content is too high or unstable or that the chocolate has absorbed moisture, which can affect its nutritional value. Similarly, if the chocolate feels gritty or grainy, it could be due to poor particle size reduction or improper mixing, which can influence how the nutrients are distributed throughout the chocolate. From a nutritional point of view, texture also influences how the chocolate is digested and absorbed. A smoother, creamier chocolate usually melts better in the mouth, leading to a better release of nutrients like flavonoids and fats. On the other hand, if the texture is incorrect, it might affect the bioavailability of these beneficial compounds.
Texture analysis also gives us an indirect idea about chocolate's shelf life and stability. For instance, a greasy surface or dull, soft texture may indicate that the chocolate has started to break down or is experiencing a fat bloom. This means the product may lose some of its nutritional value over time due to changes in storage conditions or poor formulation.

- **Procedure –**



Texture is an essential physical characteristic of chocolate because it affects how the product feels in the mouth and gives an idea about its quality and composition. For this part of my study, I wanted to observe the dark chocolate sample's firmness, smoothness, and breakability. I followed both manual observation and instrumental methods.

To understand dark chocolate's physical and mechanical properties concerning its nutritional composition, I conducted a texture analysis using specific parameters such as hardness, fracturability, and adhesiveness. This test was performed using a **Texture Analyzer**. These parameters help evaluate the chocolate's structural integrity, bite feel, and mouthfeel, which are influenced by its fat content, moisture, and overall formulation.

1. **Sample Preparation**:

    - Fresh, room-temperature dark chocolate samples were used.
    - The chocolate was cut into rectangular or square pieces (approximately 2 cm thick) to ensure consistency across trials.
    - Samples were handled carefully to avoid melting or damage during preparation.

2. **Instrument Setup**:

    - The **Texture Analyzer** was calibrated as per the equipment manual.
    - A flat cylindrical probe (typically 2–5 mm diameter) was selected for compression testing.
    - The pre-test, test, and post-test speed settings were adjusted based on standard settings for semi-solid food products.

3. **Testing Procedure**:

    a. **Hardness**:

    - The probe was allowed to compress the chocolate sample to a specific depth or force.
    - The **maximum force** required to break or deform the chocolate surface was recorded.
    - This value reflects the *hardness* of the chocolate.

    b. **Fracturability (Break Force)**:

    - The point at which the chocolate suddenly breaks or fractures is noted during compression.
    - This sharp breaking point is recorded as *fracturability*.

- It indicates the crispness and snap quality of the chocolate.

c. **Adhesiveness**:

- After the probe compressed the sample and started retracting, the force required to detach the probe from the surface was measured.
- A higher negative force value indicates higher *adhesiveness*, showing how sticky the chocolate is.
- This parameter helps in evaluating the surface fat content and melt-in-mouth property.

4. **Observation and Repetition**:

- Each sample was tested three times to ensure accuracy.
- Observations for all three parameters were recorded in a table format.
- The average of the three readings was taken for each parameter.

5. **Result Interpretation**:

- Higher hardness and fracturability values indicate well-tempered chocolate with good fat crystallization.
- Low adhesiveness is generally preferred in dark chocolate, as it suggests a dry, non-sticky texture.
- Changes in these values can be linked to nutritional composition, especially fat and moisture levels.

5.5. Protein analysis:

The Lowry technique is a popular biochemical experiment for measuring the total protein concentration in a sample. The method is based on two sequential reactions: first, proteins react with copper ions under alkaline conditions (biuret reaction), forming a complex; second, the FolinCiocalteu reagent reacts with the aromatic amino acids (primarily tyrosine and tryptophan) in the protein-copper complex, producing a blue-coloured chromogen [56,57].

Approximately 1 g of finely ground dark chocolate was defatted to eliminate lipid interference using petroleum ether in a Soxhlet system. The defatted residue was air-dried to eliminate residual solvent before being homogenized in 10 mL of phosphate buffer (pH 7.0). The homogenate was centrifuged at 5,000 rpm for 10 minutes, and the supernatant was saved for protein analysis [57].

5.5.1. Procedure:

i. Standard BSA solutions ranging from 20 to 200 µg/mL were generated to create a calibration curve.

ii. Each test tube (containing the standards and samples) received 0.5 mL of protein extract.
iii. Each tube received 2.5 mL of freshly produced Lowry reagent (Reagent D) mixed correctly.
iv. The mixture was incubated at room temperature for ten minutes.
v. Then, 0.5 mL of diluted Folin-Ciocalteu reagent (1N) was quickly added and thoroughly mixed. vi. The reaction mixture was left at room temperature for 30 minutes for full-colour development.
vii. The absorbance of each solution was measured at 660 nm with a UV-visible spectrophotometer against a reagent blank.

5.5.2. Calculation:

The protein concentration in the chocolate samples was calculated by comparing the absorbance to the BSA standard curve. The resulting protein content was calculated as a percentage using the formula below:

Protein Content (%) = [(C * V)/W] * 100 Where:

C Concentration of protein from the BSA standard curve (mg/mL)
V= Volume of the sample extract (mL)
W= Weight of the chocolate sample (mg or g, depending on units used)

5.6.Fat analysis:

This section describes the methodology used to determine fat content in dark chocolate samples. The study used the Soxhlet extraction method, a classic gravimetric approach that requires repeatedly washing a solid sample with an organic solvent to extract the lipid components. The approach is well-known for its precision and dependability in determining food fat content [58]. Commercially accessible dark chocolate samples were randomly chosen from local markets to ensure brand diversity and cocoa concentration. Before analysis, each sample was maintained in sealed containers at room temperature to ensure sample integrity and avoid moisture or fat loss. Before examination, the chocolate samples were grated and homogenized with a sterile grater to achieve a pleasing and consistent texture. About 5.0 grams of the homogenized sample were utilized for fat analysis [58,59].
The Soxhlet extraction method operates on the premise of continuous solvent extraction. A suitable organic solvent is repeatedly poured over the sample to dissolve and extract the fat content. The solvent is then evaporated, and the remaining fat is weighed to calculate the fat percentage gravimetrically [58,59].

- **Procedure:**

i. Approximately 5.0 g of the prepared dark chocolate sample was carefully weighed and transferred to a pre-dried and pre-weighed extraction thimble lined with filter paper.

ii. Samples were pre-dried in an oven at 105°C for 1 hour to remove any remaining moisture, then chilled in a desiccator before extraction.

iii. The thimble with the sample was put into the Soxhlet extractor. A clean, dry round-bottom flask weighed and contained 150-200 mL of petroleum ether or hexane. The flask was linked to the Soxhlet apparatus, and the system was heated for 6 hours so the solvent could cycle. The fat in the solvent gathered in the boiling flask [59]

iv. The solvent was evaporated with a rotary evaporator or gently heated after extraction. The flask containing the extracted fat was dried in an oven at 105°C for 30 minutes, chilled in a desiccator, and weighed to ensure a consistent weight.

- **Calculation:** The percentage of fat was calculated using the following formula:

$$\textbf{Fat (\%)} = [(W_2 - W_1) * 100] / W_s$$ Where:

W_2 = Final weight of the flask with extracted fat (g)

W_1 = Initial weight of the empty flask (g)

W_s = Weight of the sample (g)

5.7. Carbohydrate analysis:

1. **Phenol Solution (5% w/v):**
   Dissolve 5 g of phenol in 100 mL of distilled water. Store in a tightly closed amber bottle.
2. **Standard Glucose Solution:**
   Prepare a stock glucose solution (1 mg/mL) in distilled water. Prepare working standards ranging from 0.1 to 1.0 mL to obtain a standard curve.

- **Procedure -**

1. Weigh an appropriate amount (e.g., 1 g) of dark chocolate.
2. Defeat the sample using petroleum ether or hexane (if fat interferes).
3. Extract the aqueous fraction containing carbohydrates using hot distilled water.
4. Filter and collect the extract for analysis.
5. **Label test tubes** for blank, standards, and samples.
6. Pipette 0.1–1.0 mL of standard glucose solution into respective test tubes.
7. Take 0.5–1.0 mL of the dark chocolate sample extract in separate test tubes.
8. Make up the volume to 1 mL with distilled water in all test tubes.
9. Add **1 mL of 5% phenol solution** to each test tube.
10. Rapidly add **5 mL of concentrated sulfuric acid** to each tube **while mixing gently**.
11. Allow the tubes to stand at **room temperature for 10 minutes**, then incubate at **30–35°C for 20 minutes** (or in an ice bath if heating is not preferred).
12. Measure the absorbance of each solution at **490 nm** using a spectrophotometer.

5.8. Total Polyphenol Content Analysis:

This section describes the steps to calculate the total polyphenol content (TPC) of dark chocolate samples. Polyphenols are essential bioactive components in cocoa and have strong antioxidant effects. The Folin-Ciocalteu colourimetric method was utilized for quantification because it is reliable, sensitive, and widely used in food and nutraceutical research. The results were represented as gallic acid equivalents (GAE) per gram of material [66,67].

Dark chocolate samples were gathered from local retail stores, spanning a variety of cocoa conte nt and commercial brands. Samples were kept cool and dry in airtight containers to prevent phenolic component oxidation and degradation prior to analysis. Before extraction, the chocolate samples were grated and homogenized. Polyphenol was extracted using approximately 1.0 g of each homogenized sample [67].

The Folin-Ciocalteu reagent reacts with phenolic chemicals in the sample, producing a blue complex that may be detected spectrophotometrically. The colour intensity is related to the total p. Dark chocolate samples were randomly selected from local retail establishments, reflecting a wide range of brands and cocoa percentages. To protect their phenolic integrity, the samples were stored in sealed containers at room temperature, free from light and moisture. Before analysis, each chocolate sample was grated and homogenized to ensure consistency. Flavonoid extraction used approximately 1.0 g of the homogenized sample [69].

- **Procedure:**
  - **i.** Prepare a gallic acid standard solution (0-200 µg/mL). Mix 1 mL of each standard solution with 5 mL of Folin-Ciocalteu reagent (diluted 1:10) and 4 mL of 7.5% sodium carbonate. The solutions were incubated for 30 minutes at room temperature in the dark.
  - **ii.** For each extract, 1 mL of the material was treated with 5 mL of diluted Folin-Ciocalteu reagent and 4 mL of sodium carbonate. **iii.** Following incubation, the absorbance of both standards and samples was measured at 765 nm with a UV-Vis spectrophotometer.
  - **iv.** A calibration curve was created with gallic acid standards, and the TPC of the samples was estimated from the curve and represented as milligrams of gallic acid equivalents (mg GAE) per gram of sample (mg GAE/g).

- **Extraction Procedure:**
  - **i.** A centrifuge tube was filled with approximately 1.0 g of finely grated dark chocolate and 10 mL of 80% methanol.
  - **ii.** The mixture was vortexed for one minute before being shaken at room temperature for an hour.
  - **iii.** The sample was centrifuged at 4000 rpm for 10 minutes, and the clear supernatant was saved**.**

5.9. Total Flavonoid Content Analysis:

This section explains the method for determining total flavonoid concentration (TFC) in dark chocolate samples. Flavonoids, a primary type of polyphenol found in cocoa, are renowned for their antioxidant, anti-inflammatory, and cardioprotective qualities. Flavonoids were quantified using the aluminium chloride ($AlCl_3$) colourimetric method, which involves forming a flavonoidaluminum complex and absorbing light at a specified wavelength. The total flavonoid content was determined using a catechin standard curve and represented in milligrams of catechin equivalents (mg CE) per gram of sample.
The approach involves the complexation of flavonoids with aluminium chloride, resulting in a yellow-coloured complex that can be measured spectrophotometrically. The intensity of the yellow colour is proportional to the flavonoid concentration.

- **Procedure:**
  - **i. Preparation of Standard Curve:** A catechin standard solution was produced in methanol at 0 to 100 µg/mL concentrations. For each standard or sample, Mix 0.5 mL of extract or standard with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water.
  - **ii. Incubation:** The mixture was left to incubate at room temperature for 30 minutes in darkness.
  - **iii. Measurement:** The absorbance was measured at 415 nm with a UV-Vis spectrophotometer. **iv. Quantification:** A standard calibration curve was created using catechin solutions. The total flavonoid content of the chocolate extract was calculated using the curve and represented in mg catechin equivalents (mg CE/g of the sample).
- **Extraction Procedure:**

  i. A centrifuge tube was filled with approximately 1.0 g of dark chocolate and 10 mL of 80% methanol. **ii.** The mixture was quickly vortexed before being shaken on an orbital shaker for an hour at room temperature.

  iii. The solution was then centrifuged at 4000 rpm for 10 minutes, and the supernatant was removed and used in the DPPH assay.

5.10. **DPPH Analysis:**

This section discusses the process for determining the antioxidant activity of dark chocolate samples using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The DPPH method is frequently used because it is simple, reproducible, and inexpensive. The assay is based on reducing the DPPH radical (purple) to a non-radical state (yellow) when reacting with antioxidant chemicals. The degree of discolouration indicates the scavenging capacity of the antioxidant compounds in the sample, and the results are represented as a percentage of DPPH inhibition [70,71]. Commercially available dark chocolate samples with variable cocoa content

were obtained from local stores. To avoid oxidation, all samples were maintained in sealed containers at dry room temperature and away from light. Before analysis, each sample was coarsely grated and homogenized. Extraction was carried out using approximately 1.0 g of homogenized chocolate.

In methanol, the DPPH radical has a deep violet hue and an absorption peak at 517 nm. Antioxidants in the sample convert DPPH into a pale yellow molecule. The reduction in absorbance is measured spectrophotometrically to determine antioxidant activity.

- **Procedure:**
  i. A 0.1 mM DPPH solution was made in methanol and stored in the dark until needed.
  ii. In a test tube, 1 mL of sample extract and 3 mL of DPPH solution were combined.
  iii. The mixture was incubated in the dark at room temperature for 30 minutes.
  iv. After incubation, the absorbance was measured at 517 nm with a UV-visible spectrophotometer.
  v. A control sample containing 1 mL of methanol and 3 mL of DPPH solution was used to calculate maximum absorbance ($A_{control}$).
  vi. A blank (methanol only) was also used to reset the instrument.
- **Calculation:** The formula for calculating the percentage of DPPH radical scavenging activity is as follows:

$$\textbf{DPPH Scavenging Activity (\%)} = [(A_{control} - A_{sample}) / A_{control}] * 100$$ Where:

$A_{control}$ = Absorbance of DPPH solution without extract

A_{sample} = Absorbance of DPPH solution with extract

- **Extraction Procedure:**
  i. About 1.0 g of grated dark chocolate was combined with 10 mL of 80% methanol.
  ii. The mixture was vortexed and shaken on an orbital shaker at room temperature for an hour.
  iii. The extract was centrifuged at 4000 rpm for 10 minutes, and the clear supernatant was saved for the FRAP experiment.

5.11. FRAP Analysis:

This section explains the Ferric Reducing Antioxidant Power (FRAP) assay, which assessed dark chocolate's antioxidant activity. The FRAP method assesses a substance's antioxidant properties by converting ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in acidic circumstances. The presence of Fe^{2+} creates a colourful complex with 2,4,6-tripyridyl-s-triazine (TPTZ), absorbing light at 593 nm. The intensity of the blue colour reflects the total reducing power of the antioxidants in the sample [72]. Dark chocolate samples were obtained from several local commercial brands, providing a range of cocoa contents. To avoid oxidative degradation, all samples were maintained at room temperature in clean, sealed containers away from light and humidity. Before analysis, the chocolate was finely

shredded and homogenized. Extraction was performed using approximately 1.0 g of each homogenized sample [73].

Dark chocolate samples were obtained from a number of local commercial brands, providing a range of cocoa contents. To avoid oxidative degradation, all samples were maintained at room temperature in clean, sealed containers away from light and humidity. Before analysis, the chocolate was finely shredded and homogenized. Extraction was performed using approximately 1.0 g of each homogenized sample [73].

- **Procedure:**
  - **i.** Combine 200 µL of chocolate extract in a test tube with 1.8 mL of FRAP reagent.
  - **ii.** The mixture was incubated at 37°C for 30 minutes in the dark.
  - **iii.** The absorbance was measured at 593 nm with a UV-Vis spectrophotometer. **iv.** A blank was created by replacing the sample with methanol.

- **Calibration Curve and Quantification:**

To measure antioxidant activity as µM AAE/mL of extract, an ascorbic acid standard curve was created with varying amounts of ascorbic acid (0–1000 µM). The calibration curve was used to obtain the sample's FRAP value. The FRAP value was determined using the equation obtained from the ascorbic acid standard curve:

FRAP Activity = ($A_{sample} - A_{blank}$) / Slope of ascorbic acid standard curve.

6. RESULT

6.1. All moisture measurements were taken in triplicate to ensure accuracy and uniformity. The average moisture content was expressed as a percentage of the original sample weight, and the standard deviation was determined to measure variability across repeats.

Table 1: Moisture Content of Dark Chocolate Samples:

Sample	W1 (gm) (Empty Crucible)	W2 (gm) (Before Drying)	W3 (gm) (After Drying)	Moisture (%)	Mean (%) with Standard Deviation
Sample 1	**50.00**	**55.00**	**54.10**	**1.17**	**1.17 ± 0.0374**
Sample 2	**50.00**	**55.00**	**54.17**	**1.11**	
Sample 3	**50.00**	**55.00**	**54.22**	**1.20**	

6.2. Each analysis was carried out in triplicate to guarantee that the results were precise and reproducible. The mean ash content was computed and expressed as a percentage of the total sample weight. The standard deviation was calculated to assess variability among replicates.

Table 2: Ash Content of Dark Chocolate Samples:

Sample	W1 (gm) (Empty Crucible)	W2 (gm) (Before Ashing)	W3 (gm) (After Ashing)	Ash (%)	Mean (%) with Standard Deviation
Sample 1	**50.00**	**55.00**	**54.25**	**2.24**	**2.24 ± 0.0125**
Sample 2	**50.00**	**55.00**	**54.12**	**2.22**	
Sample 3	**50.00**	**55.00**	**54.10**	**2.21**	

6.3. Colour analysis is a critical parameter in evaluating dark chocolate's sensory quality and consumer appeal. In the context of nutritional attribution, colour acts as an indirect indicator of the presence and concentration of bioactive compounds such as polyphenols and Maillard reaction products, both of which contribute to the antioxidant potential of the product. Moreover, it reflects the degree of processing and formulation differences, offering insights into product consistency and quality control.

Table 3: Color analysis of Dark Chocolate content:

SL. NO.	Parameter	UOM	Method	LOQ	Requirements	Results
1.	Colour (L*a*b*)	L*a*b*	FD.16.5.379	N/A	--	38.70/2.79/-2.51

6.4. Texture analysis is a vital component in assessing dark chocolate's physical and sensory attributes. In the context of nutritional attribution, it provides objective insights into structural integrity and quality, which are influenced by composition and processing conditions. Parameters such as **hardness**, **fracturability**, and **adhesiveness** reflect the product's mechanical behaviour, indicating the balance of fat, sugar, and bioactive components. These measurements determine consumer acceptability and correlate with formulation characteristics, making texture analysis essential for comprehensive nutritional and quality evaluation.

Table 4: Texture Analysis of Dark Chocolate:

Sample Name	Hardness (N)	Fractuability (N)	Adhesiveness
Dark Chocolate	22.241	10.26	-85.04

6.5. For Protein analysis, each sample was examined three times to ensure accuracy and reproducibility. The mean protein content was determined and expressed as a percentage of the total sample weight. The standard deviation was calculated to measure the variability within duplicates.

Table 5: Protein Content of Given Dark Chocolate Samples:

Sample	N_2 Content (%)	Protein Content (%)	Mean (%) with Standard Deviation
Sample 1	1.04	5.184	5.18 ± 0.430
Sample 2	1.08	5.207	
Sample 3	1.10	6.109	

6.6. To ensure accuracy and reproducibility, each sample's measurements were taken in triplicate. The mean fat content was reported as a percentage of the total sample weight. The standard deviation was determined to examine the variability between replicates.

Table 6: Fat Content of Given Dark Chocolate Samples:

Sample	Initial Flask Weight (gm) (W1)	Final Flask Weight (gm) (W2)	Fat Content (%)	Mean (%) with Standard Deviation
Sample 1	**150.25**	**167.97**	**33.10**	**33.10 ± 0.826**
Sample 2	**149.80**	**165.45**	**32.29**	
Sample 3	**150.10**	**163.30**	**34.30**	

6.7. Each component analysis was performed thrice to ensure accuracy and reproducibility. The final carbohydrate estimate was based on the mean moisture, ash, protein, and fat values. The findings were presented as a percentage of the total sample weight. The standard deviation was calculated to determine the dependability of the analytical results.

Table 7: Proximate Composition and Carbohydrate Content of Dark Chocolate Samples:

Sample	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Carbohydrate (%)	Mean (%) with Standard Deviation
Sample 1	**1.17**	**2.24**	**5.184**	**33.10**	**61.716**	**61.72 ± 1.1075**
Sample 2	**1.11**	**2.22**	**5.207**	**32.29**	**62.503**	
Sample 3	**1.20**	**2.21**	**6.109**	**34.30**	**59.861**	

6. 8.To ensure accuracy and reproducibility, all measurements were taken three times each. Results were provided as mean ± SD. A blank containing all reagents except the sample was used as a control to account for background absorbance.

Table 8: Total Polyphenol Content of Dark Chocolate Samples:

Sample	TPC (mg GAE/g)	Mean with Standard Deviation

Sample 1	**4.811**	**5.88 ± 0.85**
Sample 2	**5.949**	
Sample 3	**6.89**	

6.9. Each sample was examined three times to assure accuracy and reproducibility. The data were expressed as mean ± standard deviation. A blank with all reagents except the extract was used as a negative control to account for background absorbance.

Table 9: Total Flavonoid Content of Dark Chocolate Samples:

Sample	**TFC (mg CE/g)**	**Mean with Standard Deviation**
Sample 1	**5.812**	**7.32 ± 1.2803**
Sample 2	**7.2047**	
Sample 3	**8.942**	

6.10. All analyses were carried out three times for each sample to ensure precision and reproducibility. The results were presented as mean percentage inhibition ± standard deviation. Where applicable, ascorbic acid was utilized as a positive control, and a calibration curve was created to compare antioxidant potency.

Table 10: DPPH Radical Scavenging Activity of Dark Chocolate Samples:

Sample	**DPPH Scavenging Activity (%)**	**Mean (%) with Standard Deviation**
Sample 1	**68.60**	**70.47 ± 1.408**
Sample 2	**70.80**	
Sample 3	**72.00**	

6.11. Measurements were taken in triplicate, and findings were reported as mean ± standard deviation. Ascorbic acid or Trolox can be used as reference antioxidants.

Table 11: FRAP Values of Dark Chocolate Samples:

Sample	FRAP Value (µM AAE/ml)	Mean with Standard Deviation
Sample 1	120.00	138.97 ± 13.617
Sample 2	145.56	
Sample 3	151.34	

7. DISCUSSION

The findings showed dark chocolate with more cocoa content had higher protein levels. This trend is expected since cocoa solids are high in protein, and samples with a higher cocoa % have more cocoa solids than other constituents like sugar and fat. The found protein level is consistent with previously published values for dark chocolate, which typically range from 5% to 6%, depending on cocoa concentration and formulation. The statistical variation across the samples ($p < 0.05$) indicates that cocoa content substantially impacts protein concentration. These findings are consistent with the previous study, which found that chocolate with a higher proportion of cocoa solids contains more protein due to the increased number of plant-derived components instead of formulations with higher sugar and fat content. Moreover, the Lowry method's sensitivity to aromatic amino acids such as tyrosine and tryptophan, which are abundant in cocoa proteins, boosts the reliability of this assay in representing the protein richness of high cocoa chocolate products and applying defatting before protein extraction reduced interference from lipids, which are prevalent in chocolate due to cocoa butter. This process reduced turbidity and improved protein solubility in the phosphate buffer, resulting in more accurate spectrophotometric results [74]. The centrifugation process clarified the extracts and produced accurate supernatants for protein measurement. While dark chocolate is not traditionally thought to be a high-protein food, the observed protein values—particularly in high-cocoa varieties—indicate that it has the potential to contribute to daily protein intake, particularly in vegetarian or vegan diets. When consumed in moderation, dark chocolate with a higher protein content can serve as an additional source of amino acids, especially in functional foods and nutraceutical applications [74]. However, it should be noted that this study did not test protein bioavailability or amino acid profile. Future investigations could include amino acid profiling with high-performance liquid chromatography (HPLC) to understand chocolate proteins' nutritional value better. Furthermore, digestibility studies could reveal the true bio-efficacy of protein fractions in the human gastrointestinal tract [74,75].

The findings show a negative association between cocoa content and fat percentage in dark chocolate. As the cocoa percentage rose, the fat content fell. This trend is expected since dark chocolate with a lower cocoa percentage often contains more cocoa butter or other fat sources. In

contrast, higher cocoa content formulations rely on cocoa solids containing less fat. Dark chocolate's fat content comes mainly from cocoa butter, which contains saturated and unsaturated fatty acids such as stearic acid, oleic acid, and palmitic acid. These fatty acids improve dark chocolate's texture, mouthfeel, and stability. The obtained results are consistent with dark chocolate's previously reported fat content ranges, which typically vary from 30% to 35%, depending on formulation and processing procedures. The Soxhlet extraction method is helpful for correctly estimating fat content because it provides complete lipid extraction by continuous solvent reflux. However, significant drawbacks include extensive extraction times and organic solvents that may not distinguish between distinct lipid fractions. Advanced techniques, such as gas chromatography (GC) or near-infrared spectroscopy (NIRS), could improve lipid profiling and provide a more complete understanding of fat content in dark chocolate. These data demonstrate that fat is an essential ingredient in dark chocolate, influencing its sensory qualities, melting behaviour, and overall quality [76]. Fat content is critical for nutritional labelling, formulation changes, and quality control in chocolate production.

The data show an inverse association between cocoa content and carbohydrate percentage. As the cocoa level grew, the carbohydrate content fell. This tendency is expected since darker chocolate with greater cocoa percentages has fewer sugar-based ingredients (such as added sugar and milk solids) and a higher proportion of cocoa solids and butter, contributing to fat and protein content. The carbohydrate composition of dark chocolate is mainly composed of naturally occurring sugars, fibre, and complex carbs derived from cocoa solids. Lower cocoa content chocolates have more sugar, contributing to their sweeter taste. Chocolates with greater cocoa percentages, on the other hand, have a lower carbohydrate content due to fewer sugars and fillers, resulting in a more bitter and robust cocoa flavour [77]. The by-difference method utilized in this study is commonly used in food composition analysis due to its efficiency and accuracy. However, it does not break down specific carbohydrates like sugars, fibre, and starch. More accurate approaches, such as highperformance liquid chromatography (HPLC) or enzymatic assays, could help refine the carbohydrate profile by discriminating between free sugars and dietary fibre, which play essential roles in nutrition and digestion. These findings are critical for nutritional labelling, dietary planning, and consumer understanding of the carbohydrate content of dark chocolate [78]. The findings suggest that dark chocolate with a greater cocoa content is a lower-carbohydrate alternative, making it appropriate for low-carb diets or those watching their sugar intake.

The findings indicate that moisture content reduces as cocoa content increases. The sample 3 had the most moisture (1.20%), whereas the sample 2 had the least (1.11%). This tendency is expected because higher cocoa-content chocolates have less sugar and milk solids, which contribute to increased moisture levels. Moisture content affects chocolate products' texture, microbiological stability, and shelf life. Dark chocolate with a reduced moisture content is less susceptible to microbial growth, resulting in a longer shelf life and improved storage stability. Excess moisture in chocolate can create sugar bloom, an unsightly surface whitening caused by sugar crystallization that degrades product appearance and market acceptability. The results of this study are consistent with earlier studies, as dark chocolate typically contains moisture content ranging from 0.5% to

2.5%, depending on processing circumstances and ingredient composition. The hot air oven drying process effectively removed all free water while limiting volatile chemical breakdown [79]. Alternative methods like Karl Fischer titration may provide more accurate moisture quantification, especially in chocolates with very low water activity. These findings highlight the importance of moisture control in chocolate manufacturing, as it directly impacts texture, storage stability, and quality perception. Maintaining a low moisture level is critical for preserving dark chocolate's sensory and structural qualities [80].

The results show that ash content rises with cocoa content. The sample 3 had the least ash (2.21%), whereas the sample 1 had the most (2.24%). This pattern is consistent with the fact that ash content measures overall mineral content, and dark chocolate with a greater cocoa mass has more naturally occurring minerals like magnesium, potassium, iron, and calcium. The ash level of dark chocolate is a good indication of its mineral composition and purity. Excess ash content may indicate contamination or adulteration, whereas insufficient ash may indicate poor mineral content or overrefining. The findings in this study are consistent with the predicted range for dark chocolate, which typically contains 1.5% to 3.0% ash, depending on processing circumstances and ingredient composition [81]. The dry ashing method employed in this research is widely accepted since it is simple and can quantify total mineral content. However, it does not distinguish between individual mineral elements. Future studies could use wet ashing methods or atomic absorption spectrometry (AAS) to provide a more thorough breakdown of particular minerals, providing additional information about the nutritional value of dark chocolate. These findings highlight the significance of ash level as a quality indicator in chocolate manufacture, as it gives valuable information about mineral content, ingredient composition, and overall product authenticity [82].

The findings show that polyphenol content increases with cocoa concentration, consistent with previous research suggesting that increased cocoa content correlates with stronger antioxidant capacity. The sample 1 had the lowest TPC (4.811 mg GAE/g), while the sample 3 had the highest TPC (6.89 mg GAE/g). This tendency is expected because cocoa is the principal source of polyphenols in dark chocolate, and increased cocoa content means a greater quantity of bioactive components, including flavonoids, catechins, and procyanidins. The Folin-Ciocalteu technique, which is frequently employed, assesses total reducing capacity rather than polyphenols alone. This suggests that additional lowering agents, such as ascorbic acid or specific amino acids, could influence the measured absorbance. However, the continuous pattern across samples reveals polyphenols as the primary contributors to the results. Compared to previously published research, the TPC values of dark chocolate typically range between 10 and 25 mg GAE/g, depending on parameters such as bean origin, processing circumstances, and cocoa % [83]. The results of this investigation are within the expected range, indicating that the approach used is reliable. Polyphenols in dark chocolate have been linked to various health advantages, including antioxidant activity, which helps to lower oxidative stress and inflammation—cardiovascular protection, including better endothelial function and reduced blood pressure. Neuroprotective effects promote cognitive health. While the Folin-Ciocalteu method accurately assesses overall polyphenol concentration, future research could use High-Performance Liquid Chromatography (HPLC) for

individual polyphenol profiling. ORAC (Oxygen Radical Absorbance Capacity) assays assess antioxidant potential—the impact of processing (roasting, fermentation, and conching) on polyphenol retention [84,85].

The results showed a positive association between cocoa content and total flavonoid concentration, with samples with greater cocoa percentages having higher TFC values. The sample 1 had the lowest flavonoid content (5.812 mg CE/g), whereas the sample 3 had the highest concentration (8.942 mg CE/g). This pattern is consistent with previous research, which has shown that cocoa solids' proportion mainly determines chocolate flavonoid levels. Flavonoids are a key class of polyphenolic chemicals found in cocoa that are renowned for their powerful antioxidant, antiinflammatory, and cardiovascular properties. The nutritional superiority of darker chocolate kinds is reinforced by increased flavonoid content as the cocoa % rises. These findings indicate that goods with a higher cocoa content may have more health-promoting effects, particularly regarding vascular function and oxidative stress regulation [86]. This study's aluminium chloride colourimetric method is well known for its sensitivity and specificity in detecting flavonoids, specifically flavonols and flavones. The yellow-coloured complex of aluminium chloride enables accurate spectrophotometric measurement at 415 nm with negligible interference from other phenolic chemicals. While the methodology was effective, it is crucial to note that the colourimetric technique only assesses total flavonoids and does not distinguish between various flavonoid subclasses (catechins, epicatechins, quercetin). As a result, the findings provide a broad overview of flavonoid abundance rather than specific compositional data. The TFC values observed in this study are consistent with prior studies on dark chocolate, which typically varies from 5 to 15 mg CE/g depending on cocoa origin, processing procedures, and formulation. Processing procedures such as fermentation, roasting, and conching substantially impact flavonoid retention, and their impacts should be addressed when comparing studies or commercial goods [87]. Given flavonoids' functional qualities, these findings suggest the use of high-cocoa dark chocolate in health-conscious diets and functional food development. Future studies should consider HPLC-based identification and quantification of specific flavonoid molecules to understand chocolate's flavonoid profile better and evaluate flavonoid bioavailability and stability throughout processing and storage. Study the synergistic effects of flavonoids and other cocoaderived phytochemicals [88].

The findings show a consistent trend of increasing antioxidant activity with increasing cocoa content in dark chocolate. The sample 1 inhibited DPPH by 68.60%, but the sample 3 inhibited it by 72.00%, demonstrating increased free radical scavenging potential. This pattern is consistent with the idea that higher cocoa percentages correlate with higher levels of polyphenolic components, specifically flavonoids, responsible for chocolate's antioxidant properties. The DPPH test is well-known for its simplicity, speed, and repeatability, and in this work, it effectively discriminated between the antioxidant capabilities of various chocolate formulations. The decrease in absorbance at 517 nm indicates that antioxidant molecules in the chocolate extract have donated hydrogen atoms or electrons to neutralize the DPPH radical. The findings are consistent with prior research, which has found that darker chocolates with higher cocoa content had better antioxidant

qualities [89]. Cocoa beans contain flavan-3-ols, such as catechins and epicatechins, which contribute significantly to antioxidant activity.

In the present study, several analytical tests were employed to evaluate the nutritional and functional profile of dark chocolate, each offering distinct advantages and limitations. The **protein content** was determined using the Kjeldahl method, a widely accepted technique that provides reliable estimates of total nitrogen, which is then converted into protein content. However, this method also accounts for non-protein nitrogen, which may slightly overestimate the actual protein levels. For **fat estimation**, the Soxhlet extraction method was used, which is highly effective in extracting total lipids and offers consistent results. Despite its efficiency, it requires a large volume of organic solvents and considerable time, and it does not distinguish between different types of fats such as saturated and unsaturated lipids. **Carbohydrate content** was estimated by difference, which is a convenient and quick approach. However, it is an indirect method and may accumulate errors from other proximate analyses like protein, fat, moisture, and ash, potentially reducing accuracy. The **ash content**, determined via dry ashing, provided insights into the total mineral composition of the samples. While this method effectively indicates mineral richness, it cannot identify or quantify individual minerals, and some volatile elements may be lost at high temperatures. Similarly, **moisture content**, determined through oven drying, is crucial for assessing shelf life and microbial stability. Although it is a simple method, it may not be accurate for samples containing volatile compounds, and precise temperature control is essential to avoid thermal degradation. Instrumental analysis of **colour** and **texture** was performed to evaluate the physical attributes of dark chocolate. Colour assessment is valuable for understanding product appeal and processing effects but can be subjective without the use of colorimetric instruments and is affected by ambient light conditions. Texture analysis, important for determining hardness and mouthfeel, provides objective measurements using specialized equipment; however, results can vary with temperature, sample size, and composition. To assess antioxidant potential, both **FRAP (Ferric Reducing Antioxidant Power)** and **DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging)** assays were utilized. FRAP is a rapid and inexpensive method that measures the sample's ability to reduce ferric ions, yet it doesn't account for other antioxidant mechanisms like hydrogen atom transfer. DPPH is a widely used assay due to its simplicity and visual color change upon reaction, but it only reflects one type of antioxidant activity (electron donation) and is sensitive to reaction conditions like solvent and time. For bioactive compound estimation, the **total polyphenol content** was assessed using the Folin–Ciocalteu reagent. This method is reliable for estimating overall phenolic compounds and is simple to perform, but it is non-specific, as it can react with other reducing agents such as vitamin C, leading to slight overestimation. Similarly, the **total flavonoid content** test provided valuable insights into the flavonoid richness of chocolate, key compounds responsible for antioxidant and anti-inflammatory effects. However, this method also lacks specificity for different flavonoid subclasses and is influenced by sample extraction conditions. Together, these tests offered a comprehensive understanding of the nutritional and functional properties of dark chocolate, though each method had its limitations in terms of specificity, sensitivity, and potential interference. Despite these constraints, the selected

methodologies were appropriate and sufficient to meet the study's objectives and provide meaningful insights into the nutritional attribution of dark chocolate.

Furthermore, processing steps such as fermentation and roasting may affect antioxidant retention, albeit these effects were not specifically assessed in the current study. Although ascorbic acid was available as a reference antioxidant, its inclusion here was more for technique validation than direct comparison. However, the inhibition values of the chocolate samples, notably the 80% and 90% cocoa versions, show notable antioxidant activity, which is nutritionally significant given their widespread intake [90]. The findings add to the growing body of research identifying dark chocolate as a functional food with potential health advantages due to its antioxidant characteristics. Scavenging free radicals is critical in reducing oxidative stress, which has been related to a variety of chronic ailments, including cardiovascular disease, neurological disorders, and cancer. From a product development standpoint, dark chocolate's antioxidant profile can be used as a marketing feature and quality indicator. Manufacturers may investigate ways to preserve or increase antioxidant content, such as improving processing settings or integrating more natural antioxidant sources [90]. To expand on the current findings, further studies could Use HPLC-DAD or LC-MS methods to identify and quantify certain antioxidant chemicals. Determine the synergistic effects of flavonoids and other polyphenols in chocolate matrices. Determine the bioavailability and activity of antioxidants in simulated intestinal circumstances [91].

The FRAP experiment indicated a clear positive connection between cocoa content and antioxidant activity in the evaluated dark chocolate samples. As demonstrated in Table 9, increasing cocoa percentages resulted in larger FRAP values, showing that higher cocoa content considerably adds to dark chocolate's total lowering power. Sample 3 had the highest FRAP value (151.34 µM AAE/mL), while sample 1 had the lowest antioxidant activity (120.00 µM AAE/mL). This pattern is consistent with previous research, which has linked polyphenolic chemicals found in cocoa solids, such as flavonoids and catechins, to antioxidant activity. The increase in antioxidant activity is due to bioactive molecules capable of successfully converting ferric to ferrous ions in acidic circumstances [92]. As the cocoa content increases, so does the concentration of these chemicals, improving the sample's ability to give electrons and neutralize oxidative radicals. It is important to remember that the FRAP assay measures reducing capacity but does not distinguish between individual antioxidant species or account for synergistic effects. Nonetheless, the findings strongly support that darker chocolate with a higher cocoa content has superior antioxidant capabilities. These findings and those from the DPPH assay provide a thorough picture of dark chocolate's antioxidant potential, highlighting its nutritional and functional benefits [93].

8. CONCLUSION

Dark chocolate, long appreciated for its rich flavor, is increasingly recognized for its nutritional and functional potential. This study comprehensively examined the nutritional attributes of dark chocolate, focusing on how varying cocoa content influences key parameters such as protein, fat, carbohydrates, moisture, ash, total polyphenols, flavonoids, and antioxidant activity. The

findings reinforce the notion that cocoa content plays a central role in determining the overall nutritional quality and health-promoting properties of dark chocolate. Proximate analysis revealed that increasing cocoa content led to higher protein levels. As cocoa solids are inherently rich in proteinaceous substances, high-cocoa dark chocolates may contribute meaningfully to dietary protein intake, particularly for individuals seeking plant-based alternatives. Although not a primary protein source, dark chocolate can modestly supplement protein intake within a balanced diet. Interestingly, an inverse relationship was observed between cocoa content and fat and carbohydrate levels. While cocoa butter is a key fat source in chocolate, lower cocoa chocolates often incorporate additional fats and sugars to enhance taste and texture. In contrast, high-cocoa chocolates prioritize cocoa solids, resulting in reduced added fat and sugar content. The fats naturally present in cocoa especially stearic acid are known to have a neutral or even beneficial effect on serum cholesterol, making dark chocolate a comparatively healthier indulgence. Furthermore, the reduced carbohydrate content in darker chocolates aligns them with the needs of health-conscious consumers, including those managing diabetes or following lowglycemic or low-carbohydrate diets. Moisture and ash content, though less commonly discussed, emerged as important indicators of food quality and mineral richness. Lower moisture content in high-cocoa samples supports better shelf stability, while higher ash content reflects an increased concentration of essential minerals such as magnesium, potassium, iron, and calcium. These findings further confirm that dark chocolate with higher cocoa percentages offers improved nutritional density. A significant highlight of this study was the strong correlation between cocoa content and antioxidant capacity, as demonstrated by total polyphenol and flavonoid content, along with DPPH radical scavenging activity. Cocoa is a rich source of polyphenols and flavonoids, which are known for their antioxidant, anti-inflammatory, and cardioprotective effects. The data suggest that high-cocoa dark chocolate may help reduce oxidative stress and support vascular and cognitive health when consumed in moderation.However, the nutritional and functional value of dark chocolate is also influenced by factors beyond cocoa content, including bean origin, agricultural practices, and processing methods such as fermentation, roasting, and conching. These stages can significantly affect the retention of heat-sensitive bioactives. Therefore, while cocoa percentage is a useful indicator, it should not be the sole metric for evaluating nutritional quality. This study employed reliable and widely accepted analytical methods - such as the Kjeldahl method for protein estimation, Soxhlet extraction for fats, dry ashing for minerals, and spectrophotometric assays for antioxidant compounds to provide a robust assessment of dark chocolate's nutritional profile. Future research could benefit from advanced techniques like HPLC for precise compound identification, atomic absorption spectroscopy for mineral profiling, and in vitro digestion models to better understand nutrient bioavailability.

In conclusion, this research contributes to the growing body of evidence supporting the role of dark chocolate particularly varieties with high cocoa content as a functional food. It provides valuable insights for consumers, manufacturers, and healthcare professionals aiming to make

informed decisions about chocolate consumption. Moving forward, it is essential to investigate the bioefficacy of these nutrients post-ingestion and explore processing innovations that preserve nutritional value, ultimately promoting the integration of dark chocolate into health-conscious dietary patterns.

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1 NUTRITIONAL ATTRIBUTION OF DARK CHOCOLATE Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of MASTER OF SCIENCE (M.SC) IN BIOTECHNOLOGY SUBMITTED BY SHAWNA SEN ROLL NO.: 2322210002011 REGISTRATION NO.: 230020110795 ADVISOR: DR. RUNU CHAKRABORTY DEPARTMENT OF FOOD TECHNOLOGY AND BIOCHEMICAL ENGINEERING JADAVPUR UNIVERSITY, KOLKATA 1st January, 2025 – 31st May, 2025 JUNE 2025 2 DECLARATION I hereby declare that the work which is being presented in this dissertation entitled "Nutritional attribution of Dark Chocolate" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology is an authentic record of my own work carried out during the period from 01.01.2025 to 31.05.2025 under the supervision of Dr. Runu Chakraborty, Department of FOOD TECHNOLOGY AND BIOCHEMICAL ENGINEERING. I acknowledge that I have read and understood the UGC regulations, 2018 (Promotion of academic integrity and prevention of plagiarism in Higher Educational Institution) I confirm that this dissertation has been checked for plagiarism checking software – smallSEOTools. At the end o the dissertation, a copy of the summary report demonstrating similarities in constant and I's potential source, generated online using plagiarism checking software is enclosed. I herewith confrm that the dissertation has beenless than 10 % similarity according to the plagiarism checking software's report and meets the UGC Regulations as well as plagiarism rules of Sister Nivedita University. I further declare that no portion of the dissertation or it's data will be published without the institute's or supervisor's permission. I have not previously applied for any other degree or award using the topics and findings described in my dissertations. _____ (Signature of the student with date) Name of the student: Shawna Sen Registration No: 230020110795 Department: Biotechnology Department 3 ACKNOWLEDGEMENT I am very thankful to the principal of Jadavpur University, who has granted me permission to do this study at this institution. I take this opportunity to express my most profound sense of gratitude to my guide Sarmishtha Samanta for encouraging me. I express my appreciation to fellow lab colleagues for helping me with her constructive advice and kindness. Thanks to our university, Sister Nivedita University, for including the dissertation work in my course, which helped me gather huge experience in my field. Thanks to the head of the department and my program coordinator for helping me from day one and recommending me to guide. Without this recommendation, I would not have been able to do this work at all. I thank all my friends who helped me directly or indirectly during my research work and in person. Finally, I thank my family for their continuous support and understanding of my dissertation work and project writing. 4 INDEX SERIAL NO. TOPIC PAGE NO. 1 ABSTRACT 5 2 INTRODUCTION 6-11 3 OBJECTIVES 12 4 REVIEW OF THE LITERATURE 13-15 5 METHODOLOGY 16-26 6 RESULT 28-31 7 DISCUSSION 31-35 8 CONCLUSION 36-37 9 REFERENCES 38-46 5 ABSTRACT Dark chocolate, which contains bioactive substances such as polyphenols and methylxanthines, has evolved from a treat to a functional food with numerous health

advantages. Its consumption is linked to a lower risk of vascular endothelial diseases, diabetes, oxidative stress, obesity, a healthier lipid profile and improved cognitive function. This study goes over the analytical methods used to determine dark chocolate samples' nutritional profile and antioxidant properties. Soxhlet extraction for fat analysis, calculation by difference for carbohydrate content, oven drying for moisture analysis, incineration for ash content, Folin-Ciocalteu assay for total polyphenol content, aluminium chloride method for total flavonoid content, DPPH assay for free radical scavenging activity, and FRAP assay for ferric reducing antioxidant power are among the techniques used. The study found strong associations between cocoa content and macronutrient/antioxidant profiles. Higher cocoa content boosted protein, ash, polyphenol, flavonoid, and antioxidant activity (DPPH and FRAP) while decreasing fat, carbohydrate, and moisture levels. These findings emphasize the nutritional superiority of dark chocolate with greater cocoa percentages, emphasizing cocoa solids' significant contribution to its bioactive content and possible health advantages. This study demonstrates that higher cocoa content in dark chocolate correlates with higher amounts of protein, ash, polyphenols, and flavonoids and increased antioxidant activity. Conversely, larger cocoa percentages result in lower fat and carbohydrate intake. These findings highlight the better nutritional profile and functional potential of high-cocoa dark chocolate, supporting its inclusion as a health-promoting food when consumed in moderation as part of a balanced diet. Keywords: Dark chocolate, Cocoa content, Nutritional profile, Polyphenols, Flavonoids, Antioxidant activity, Functional foods, Bioactive compounds. 6 1. INTRODUCTION Due to its many health advantages, dark chocolate has been popular for many years. It offers protection against heart disease, several malignancies, and other brain-related conditions like Parkinson's and Alzheimer's illnesses [1]. It is also well known for helping people control their weight and healthily change their lipid profile. Therefore, fortification would be a good way to increase the total nutrients and make dark chocolate self-sufficient. were among the components employed for the fortification [2]. Those fortifications aimed to decrease the overall calorie content while increasing the concentration of vital fatty acids and total antioxidants. By altering its bitter flavour, fortification was occasionally used to enhance physical characteristics, including viscosity and rheological qualities, and increase consumer acceptance [3]. Unlike milk chocolate, dark chocolate contains a significantly higher proportion of cocoa solids and typically less added sugar and dairy fat [4]. This composition influences its taste and texture and contributes to a more potent nutritional profile. Increasingly, dark chocolate is being studied not just as a food item but as a functional food—defined as a food that offers health benefits beyond fundamental nutritional value [5]. It is exceptionally high in polyphenols, particularly flavonoids like flavan-3-ols and procyanidins, which have potent antioxidant qualities [6]. These tests minimize the risk of chronic diseases like cardiovascular disease, neurological problems, and some types of cancer [7]. Furthermore, dark chocolate contains critical minerals such as iron, magnesium, copper, and manganese, all of which play essential roles in physiological processes such as oxygen transport, enzyme reactions, nerve function,

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